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SUBTITLE: Development of Safe, Effective Vaccines for Dengue Disease Utilizing Viral Envelope and NSI Glycoproteins Expressed by Recombinant Baculovirus

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FOREWORD

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Ching-Juh Lai Sep 16, '91

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INTRODUCTION

Outbreaks and major epidemics of dengue continue to afflict human populations, especially in the tropical and subtropical regions of the world. Dengue viruses, transmitted predominantly by mosquito species of the Aedes genus, include four serotypes that are distinguishable by plaque reduction--neutralization with type specific monoclonal antibodies (1,2). Dengue is characterized by fever, rash, severe headache, and joint pain. Its mortality rate is low. However, over the past few decades, a more severe form of dengue, characterized by hemorrhage and shock has been observed with increasing frequency in children and young adults. This severe form of dengue has a high mortality rate. Despite years of intensive research, an effective vaccine is still not available for prevention of dengue infection. For these reasons, research on dengue viruses and development of safe and effective vaccines have been given a high priority by the World Health Organization.

Similar to other members of the flavivirus family, dengue virus contains a positive RNA genome and three structural proteins, i.e. capsid protein (C), the small membrane protein (M), and the large membrane or envelope (E) glycoprotein. The RNA genome codes for a long polyprotein that is co- and post-translationally cleaved to produce the three structural proteins and a series of non-structural proteins designated NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 in that order.

One of our research goals has been to develop a safe and effective vaccine for prevention of dengue virus infection. Earlier, we constructed recombinant vaccinia virus that contained dengue type 4 virus cDNA coding for the C-PreM-E-NS1-NS2A polyprotein. Infection of CV-1 cells with this recombinant virus produced apparently authentic PreM, E, and NS1 glycoproteins (3). Mice immunized with this recombinant vaccinia virus were completely protected against lethal dengue encephalitis (4). This successful effort prompted us to construct recombinant baculovirus b(C-PreM-E-NS1-NS2A) that contained the same cDNA coding for polyprotein C-PreM-E-NS1-NS2A. Immunization of mice with the lysate prepared from the recombinant virus infected-insect cells induced complete or nearly complete resistance to lethal intracerebral dengue virus challenge (5). Analysis of seroresponse showed that immunized mice developed a high level of antibodies specific for NS1, whereas antibodies to E or PreM were low or not detected. In order to clarify the role of E in inducing resistance to dengue virus challenge, recombinant baculovirus b(RSVG-E) expressing only E was constructed utilizing the amino terminal 71 amino acid sequence of respiratory syncytial virus (RSV) G glycoprotein as a signal for proper glycosylation of E. Mice immunized with a lysate of insect sf9 cells infected with this recombinant virus failed to develop a detectable seroresponse to E, nevertheless, these mice were completely resistant to dengue virus challenge (6). These observations indicate that the E glycoprotein is a major

protective antigen but left open the mechanism responsible for resistance.

Immunization of mice with dengue virus NS1 alone was investigated using recombinant vaccinia virus expressing authentic NS1. In this construct the 24 hydrophobic amino acid sequence preceding NS1 and the downstream NS2A sequence were both present in order to achieve authentic synthesis of dengue virus NS1 (7). During studies in mice, this recombinant vaccinia virus induced complete protection against dengue challenge. These results showed that dengue NS1 is an independent protective antigen in mice (8). Recently, the protective efficacy of recombinant baculovirus expressed-dengue virus proteins was evaluated in rhesus monkeys. The results showed that one of three monkeys immunized with dengue virus proteins expressed by recombinant b(C-PreM-E-NS1-NS2A) was protected against dengue virus challenge. Also, only one of three monkeys immunized with dengue E alone expressed by b(RSVG-E) was completely protected (9).

Because only partial protection was observed, it is necessary to analyze the quantitative relationship between the amount of viral proteins used for immunization and the extent of protective effect. The protective efficacy of individual antigens should be separately evaluated, and if necessary, improved through genetic manipulation or addition of an appropriate carrier. A stoichiometric analysis may require greater amounts of dengue viral antigens than that can now be

produced by the available recombinants. For these reasons, during the initial phase of this study we have targeted the goal of separately expressing authentic envelope and NS1 glycoproteins. We have achieved high level production of these dengue proteins using a high yield baculovirus expressing vector. Biochemical characterization of several dengue protein products has also been completed.

RESULTS

(a) Expression of authentic dengue type 4 virus envelope and nonstructural NS1 glycoproteins

Earlier we produced a fusion dengue E glycoprotein that contained the 71 N-terminal amino acids of RSV G glycoprotein fused to amino acids 1-456 (93%) of dengue type 4 E. Immunization of monkeys with the baculovirus recombinant that expressed this dengue E fusion protein induced only partial resistance to challenge with homotypic dengue virus. Hence we felt it would be desirable to evaluate a non-fusion construct of E that expresses authentic E glycoprotein. For this purpose, the dengue DNA fragment that codes for amino acids 1-456 of E plus the predicted N-terminal hydrophobic signal sequence of E (amino acids 265-279 of the dengue 4 polyprotein sequence) preceded by a start codon was obtained from the intermediate vaccinia virus transfer vector constructed earlier (4). This DNA construct was extended to include the coding sequence for the remaining C-terminal 38 amino acids of E plus the first 4 amino acids of NS1 followed by a stop codon. The resulting DNA sequences were inserted into baculovirus plasmid vector pVL 941 (kindly provided by Dr. M. Summers, Texas A & M University, College Station, Texas). The recombinant DNA was recombined into the genome of wild-type Autographa californica mononuclear polyhedrosis virus (ACMNPV) replacing the polyhedrin coding sequence. A recombinant baculovirus was selected and purified from the wild-type ACMNPV after serial-dilution passage and visual selection of virus

plaques lacking polyhedrin. This recombinant virus expressed dengue 4 E as detected by an indirect immunofluorescence assay (IFA) using dengue hyperimmune mouse ascitic fluid (HMAF). This recombinant virus was designated b(DEN4, 100%E).

Proper synthesis of dengue virus NS1 has been studied in the laboratory using vaccinia virus as a vector. We found that the 24 hydrophobic amino acids preceding NS1 and the downstream NS2A sequences were required for authentic synthesis of dengue virus NS1 (Falgout, et al. 1989). DNA sequences coding for dengue 4 NS1-NS2A were prepared from pSC11-NS1-NS2A that had been used for construction of the recombinant vaccinia virus. The DNA fragment was inserted into the baculovirus cloning vector and recombinant plasmid pVL941 NS1-NS2A was isolated. The recombinant baculovirus that contained the DNA insert was designated b(DEN4, NS1-NS2A) was constructed and isolated from wild type baculovirus by serial dilution-passage and plaque purification. IFA was used to identify the dengue virus product expressed in recombinant-infected cells.

(b) Analysis of full-length dengue 4 E and NS1 expressed by recombinant baculoviruses

To verify the dengue protein product expressed by recombinant b(DEN4, 100%E), insect sf9 cells were infected with this recombinant, labeled with ^{35}S-methionine, and lyzed in RIPA buffer (1% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecylsulfate, 0.1M Tris-HCl, PH 7.5, 0.15M NaCl). The radio-

labeled lysate was prepared for immunoprecipitation followed by separation on SDS-polyacrylamide gel. The results showed that a labeled protein with a molecular weight of 55-60 kilodaltons (Kd) predicted for the glycosylated form of dengue E was produced.

Insect sf9 cells were also infected with b(DEN4, NS1-NS2A), radio-labeled, and the lysate prepared for immunoprecipitation using HMAF. Analysis of the immunoprecipitate by polyacrylamide gel electrophoresis revealed a protein band of 40-44 Kd predicted for the NS1 glycoprotein. This confirmed that properly cleaved dengue virus NS1 was made during infection of insect cells with the recombinant construct. The results of this analysis indicate that separate expression of dengue type 4 virus E and NS1 glycoproteins has been achieved using the high yielding baculovirus-insect cell system. This initial success has set the stage for further extension of the proposed studies concerning the production of protective antigens belonging to the remaining three dengue virus serotypes.

(c) Construction of recombinant baculovirus expressing a high level of dengue 4 NS1

Dr. H. Hori of the laboratory studied the amino acid sequence at the NS1-NS2A juncture of dengue virus that is required for specific cleavage effected by the cis-acting function of NS2A. During this study it was observed that recombinant vaccinia virus v(DEN 4, NS1) containing dengue type 4 virus cDNA that codes for the N-terminal signal and the entire

length of the NS1 sequence produced the NS1 glycoprotein at a level 2-3 fold higher than that expressed by recombinant v(DEN 4, NS1-NS2A) which contained NS2A (Hori and Lai, unpublished observation). Although the reason for the increased synthesis of NS1 is not known, we speculate that proteolytic cleavage of polyprotein NS1-NS2A may be inefficient, thus retarding the synthesis of NS1. Since it was desirable to increase the production of NS1, the DNA fragment that codes for the full-length NS1 was prepared and used for construction of a recombinant baculovirus expressing NS1 in the absence of downstream NS2A. This recombinant baculovirus, designated b(DEN 4, NS1) was isolated using the methodology just described. This recombinant DNA strategy for increased expression of dengue 4 NS1 was also adopted for construction of recombinant baculoviruses expressing the NS1 glycoproteins of other dengue virus serotypes.

(d) Construction of recombinant baculovirus expressing dengue 2 E or NS1 glycoproteins

The dengue virus subgroup of the flavivirus family consists of four serotypes (type 1 to type 4), all of which cause dengue epidemics in many tropical and subtropical areas. There is a high degree of conservation of the amino acid sequences among the four dengue viruses. Amino acid homology of E sequences among different dengue serotype viruses ranges from 62 to 70%. Likewise, amino acid homology of NS1 sequences among different

dengue viruses ranges from 66-73%. These similarities suggest that vaccine strategies developed for dengue type 4 virus should be applicable to the other three dengue virus serotypes. Also, there is a concern about immune enhancement as the underlying cause for severe dengue. Thus, the current strategy for immunization against dengue favors the use of a tetravalent vaccine that contains protective antigens of all four serotype viruses.

As an initial step in this direction, we constructed a recombinant baculovirus expressing dengue type 2 E or NS1 glycoprotein. cDNA fragments of dengue type 2 virus (S1 candidate vaccine strain derived from the PR159 isolate), cloned and sequenced in Dr. Strauss' laboratory (California Institute of Technology, Pasadena, CA), were kindly provided by Dr. Strauss through Dr. Putnak (Walter Reed Amry Institute of Research, Washington, D.C.). Clone E8 was the source for E DNA. Dr. Bray in the laboratory constructed a cDNA sequence coding for the 15 amino acid N-terminal signal and the entire length of E (495 amino acids) (10). In order to construct recombinant baculovirus, the E DNA construct was inserted into the intermediate baculovirus vector, pBlueBac (In Vitrogen Corp., San Diego, CA), which contains a <u>laz</u> sequence for visual selection of the recombinant virus. The recombinant virus, designated b(DEN2, 100%E) expressed the encoded dengue virus protein as detected by IFA.

In order to verify the expression of dengue 2 NS1, insect sf9 cells were infected with b(DEN2, NS1), labeled with ^{35}S-methionine, lysed in R1PA buffer, and the lysate prepared for analysis of dengue NS1 by immunoprecipitation. Analysis of the immunoprecipitate by polyacrylamide gel electrophoresis showed that a protein approximately 40-44 Kd of the size predicted for dengue NS1 was detected. The estimated molecular size suggested that the NS1 product was glycosylated consistent with the result obtained earlier. This study showed that recombinant baculovirus b(DEN2, NS1) expressed apparently authentic NS1 of dengue 2 virus. Similarly, analysis of dengue 4 NS1 produced by recombinant b(DEN4, NS1), another variant of recombinant baculovirus expressing dengue 4 NS1, was also completed. These studies showed that a protein band with a molecular size approximately 40-44 Kd was made in recombinant b(DEN 4, NS1) infected cells.

(e) Dengue virus NS1 expressed by recombinant baculovirus was not detected extracellularly.

It has been reported that a small fraction of dengue NS1 is secreted into the medium during dengue virus infection of cultured cells (11). We have shown earlier that extracellular dengue virus NS1 was detected at a low level (<2%) in recombinant vaccinia virus v(NS1-NS2A) infected cells, similar to that found during dengue virus infection (Falgout and Lai, unpublished observation). Extracellularly secreted NS1 migrated slower than

did the intracellular NS1 indicating that additional glycosylation had occurred during its transfer through the exocytic pathway. As part of our analysis of dengue type 2 or type 4 NS1 produced by recombinant baculovirus the medium fraction of ^{35}S-methionine labelled-infected cells was also immunoprecipitated and separated by polyacrylamide gel electrophoresis. The results showed that extracellular NS1 was not detected by immunoprecipitation.

(f) Construction of recombinant baculovirus expressing highly immunogenic E

Recent results obtained from our laboratory showed that several C-terminally truncated E's, approximately 80% in length, exhibit increased immunogenicity in mice (12). Unlike full-length E, these truncated E's were detected on the cell surface and secreted extracellularly. In addition to being more immunogenic, a significant fraction of the E product was secreted extracellularly. Extracellularly secreted 80%E also possesses an advantage over intracellular full-length E in the greater ease with which it can be purified. For these reasons, we have initiated studies to construct recombinant baculovirus for the production of 80%E truncated at the C-terminus. Initial success has been made in the construction of recombinant DNA and the generation of recombinant baculovirus. Experiments to analyze quantitatively the E product expressed by the recombinant are currently underway. We plan to evaluate the immunogenicity and

protective efficacies of the full-length and the C-terminally truncated E's in mice. The level of antibody response in immunized mice will be determined by serologic analysis. The subunit protein products that induce an increased antibody response will be further evaluated in primates which are the best available experimental surrogates for human dengue virus infection.

CONCLUSIONS

The envelope (E) and NS1 nonstructural glycoproteins of dengue virus have been identified as independent protective antigens in mice during studies in which these glycoproteins expressed singly or together by recombinant vaccinia virus or baculovirus were evaluated for their protective immunity against lethal intracerebral dengue challenge. As proposed, the research goals of this study are to; (1) produce authentically processed dengue subunit protective antigens using the high yielding baculovirus-insect cell expression system; and (2) design recombinant DNA constructs that code for highly immunogenic protein products, and achieve a high level expression of these products.

During the initial phase of this study we have completed construction of recombinant baculoviruses that separately express full-length E or NS1 glycoprotein of dengue type 2 and type 4 viruses. Radio-immunoprecipitation was performed to analyze the dengue product in the lysate of recombinant virus infected cells. The results showed that insect cells infected with recombinant b(DEN 4, 100%E) produced a protein with molecular weight of 55-60 kilodalton (Kd) as predicted for the glycosylated form of dengue E glycoprotein. Similarly, recombinant b(DEN 4, NS1) or b(DEN 2, NS1) produced a protein in infected insect cells that was approximately 40-44 Kd, the size predicted for the dengue NS1 glycoprotein. In the next phase of this study we plan to prepare lysates of insect cells infected with various recombinant

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